(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2001 (01.11.2001)

PCT

(10) International Publication Number
WO 01/80715 A2

(51) International Patent Classification⁷: A61B

(21) International Application Number: PCT/US01/12877

(22) International Filing Date: 20 April 2001 (20.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/553,927 21 April 2000 (21.04.2000) US

(71) Applicant: THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US).

(72) Inventor: TABAS, Ira; 13 Jodi Lane, New City, NY 10956 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
..... without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR IDENTIFYING COMPOUNDS USEFUL FOR PREVENTING ACUTE CLINICAL VASCULAR EVENTS IN A SUBJECT

(57) Abstract: This invention provides a method of determining whether a compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell which comprises: (a) admixing the compound with a cell; (b) contacting the mixture in (a) with a toxin that causes cell death only if excess cholesterol is present at the peripheral site; (c) determining whether the cell either is living or non-living, wherein a living cell indicates that the compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell. The invention also provides a method for preventing or delaying plaque rupture or superficial erosion in a subject which comprises administering to the subject an effective amount of a pharmaceutical composition comprising (i) a compound that inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site; and (ii) a carrier so as to prolong the life of a macrophage within plaque lesions which exist in the subject, thereby preventing or delaying plaque rupture or superficial erosion in the subject.

WO 01/80715 A2

Methods For Identifying Compounds Useful For Preventing Acute Clinical Vascular Events In A Subject

The invention described herein was made with Government support under grant number HL54591 from the National Institutes of Health. Accordingly, the United States Government has certain rights in this invention.

Background of the Invention

Throughout this application, various publications are referenced by arabic numbers within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found listed numerically immediately preceding the claims.

A prominent feature of advanced atherosclerotic lesions is the presence of necrotic areas, which are sites inside the thickened intima consisting of cellular debris and extracellular lipid (1,2). The importance of these necrotic areas lies in the fact that they are often found in areas of plaque rupture, which is the most common precipitating cause of atherosclerosis-associated acute thrombosis, vascular occlusion, and tissue infarction (3). Given the potential clinical implications of lesion necrosis, surprisingly little is

known about the mechanisms of necrotic area development. While there is evidence that some of the lipid in these areas is derived directly from extracellular, plasma-derived lipoproteins, cell death with subsequent release of intracellular lipids and other potentially harmful molecules is likely to be a central event (2,4,5). In this regard, recent data with antibodies against cell-type-specific intracellular proteins support the idea that the cholesterol-loaded macrophage, a major cellular constituent of atherosclerotic lesions, is the main cell type that dies in the vicinity of necrotic areas (4,6). The mechanistic link between macrophage death and unstable plaques may be related to plaque-destabilizing enzymes and pro-coagulant/thrombogenic molecules released by these dying cells (7).

The causes of macrophage death in advanced atherosclerosis are not known. Several factors or conditions, such as oxidized lipids, growth factor deprivation, and inflammatory cytokines, have been proposed but not rigorously tested *in vivo* (1,8). Another cytotoxic condition that deserves attention is excess cellular free cholesterol (FC) (9). FC accumulation in lesional foam cells has been well-documented (10, 11, 12, 13), and studies with cultured macrophages have shown that excess cellular FC is a potent inducer of cell death (14, 15). The mechanism of cytotoxicity probably involves integral membrane protein dysfunction resulting from a high cholesterol:phospholipid ratio in the membranes

surrounding these molecules (9, 16, 17). This idea was recently supported by the data of Kellner-Weibel *et al.* (18), who showed that FC-induced cytotoxicity was inhibited by amphipathic amines, which block the transport of lipoprotein-derived FC from lysosomes to peripheral membranes, particularly the plasma membrane. Interestingly, Papahadjopoulos (16) demonstrated twenty-five years ago that FC-mediated inhibition of the plasma membrane proteins Na^+-K^+-ATPase and adenylate cyclase leads to cellular death, and he proposed that these events may play an important role in the development of necrosis in advanced atheromata.

Although cell cultures studies have suggested potentially important ideas related to inducers and mechanisms of macrophage death, little is known about the factors that influence the development of lesional necrosis *in vivo*.

Summary of the Invention

This invention provides a method of determining whether a compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell which comprises: (a) admixing the compound with a cell; (b) contacting the mixture in (a) with a toxin that causes cell death only if excess cholesterol is present at the peripheral site; (c) determining whether the cell either is living or non-living, wherein a living cell indicates that the compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell. The invention also provides a method for preventing or delaying plaque rupture or superficial erosion in a subject which comprises administering to the subject an effective amount of a pharmaceutical composition comprising (i) a compound that inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site; and (ii) a carrier so as to prolong the life of a macrophage within plaque lesions which exist in the subject, thereby preventing or delaying plaque rupture or superficial erosion in the subject.

Brief Description of the Figures

Fig. 1. NPC1 macrophages are resistant to FC-mediated cytotoxicity. Peritoneal macrophages from wild-type and NPC1 mice were incubated for 24 h in serum-free medium alone (*cross-hatched bars*) or medium containing 10 μg acetyl-LDL/ml plus 10 μg 58035/ml (*solid bars*) to effect FC loading. The cells were then stained with propidium iodide to determine the percentage of necrotic cells.

Fig. 2. Characterization of necrotic areas in the advanced atherosclerotic lesions of E0 mice. Adjacent sections of proximal aortic lesions from 25-week-old cholesterol fed E0 mice were stained with hematoxylin (*A*), filipin (*B*), anti-type A scavenger receptor antibody (*C*), and control antibody (*D*). The *arrow* depicts one of the several sites that have the characteristics of necrotic areas.

Fig. 3. Plasma lipids and lipoprotein profile of E0 and NPC1/E0 mice. The plasma of 26 E0 mice (14 females and 12 males; *cross-hatched bars*) and 9 NPC1/E0 mice (3 females and 6 males; *solid bars*) were assayed for cholesterol and phospholipid concentrations (*A*), and pooled plasma samples from two male E0 mice (*open circles*) and two male NPC1/E0 mice (*closed circles*) were subjected to FPLC gel-filtration fractionation (*B*). The differences between the two groups of mice for both cholesterol and phospholipid levels in *panel A* were not statistically significant. The difference between the

two groups of mice in the FPLC peak around fraction #18 in *panel B* was not observed in additional experiments.

Fig. 4. Atherosclerotic lesion area and necrotic area in the proximal aorta of E0 and NPC1.E0 mice. Six sections of proximal aorta from 26 E0 mice (14 females and 12 males; *cross-hatched bars*) and 9 NPC1/E0 mice (3 females and 6 males; *solid bars*) were assayed for average atherosclerotic lesion area (*A*) and necrotic area (*B*); in *panel C*, the data are expressed as percent necrotic area ([necrotic area ÷ lesion area] x 100).

Fig. 5. Hematoxylin- and Oil Red O-stained sections of lesions from an E0 mouse and an NPC1/E0 mouse. Adjacent sections of a proximal aortic lesion from a male E0 mouse were stained with hematoxylin (*A*) or Oil Red O (*A'*). Similar staining was done for sections from a male NPC1/E0 mouse in *B* and *B'*. The *asterisks* in *panel A* depict acellular areas; these areas stained only weakly for collagen (data not shown). The *closed arrowheads* in *panel B* show cellular areas, and the *open arrow* in *panel B* shows an area containing cholesterol crystals. Note that the cellular areas stain more intensely with Oil Red O, which preferentially stains neutral lipids like cholesteryl ester.

Detailed Description of the Invention

The following abbreviations are used herein: ACAT: acyl-CoA:cholesterol acyltransferase; E0: apolipoprotein E knockout; FC: free cholesterol; LDL: low-density lipoprotein; NPC: Niemann-Pick; NPC1: heterozygous NPC knockout; PI: propidium iodide; VLDL: very low-density lipoprotein.

The present invention provides for a method of determining whether a compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell which comprises: (a) admixing the compound with a cell; (b) contacting the mixture in (a) with a toxin that causes cell death only if excess cholesterol is present at the peripheral site; (c) determining whether the cell either is living or non-living, wherein a living cell indicates that the compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell.

In one embodiment of the above method, the intracellular cholesterol storage site is a lysosome. In another embodiment, the intracellular cholesterol storage site is a recycling endosome. In another embodiment, the intracellular cholesterol storage site is a sorting endosome. In another embodiment, the intracellular cholesterol storage site is a late endosome.

In another embodiment, the peripheral site is a plasma membrane of the cell or a mitochondria, an endoplasmic reticulum, a peroxisome, nucleus or a Golgi apparatus in the cell, or any other site where a high free cholesterol content might cause cellular damage.

In another embodiment, the cell is a macrophage, an endothelial cell, a smooth muscle cell, a T cell, a dendritic cell, or any other arterial-wall cell that might play a role in atherogenesis.

In another embodiment, the toxin is amphotericin B, filipin, streptolysin O, pneumolysin, perfringolysin O (theta toxin), Vibrio cholerae cytolysin, aerolysin, Listeriolysin O, Vibrio vulnificus haemolysin (VVH), staphylococcal alpha toxin, Aeromonas hydrophilia cytotoxic endotoxin (ACT) or any derivative thereof, or any compound or derivative thereof that causes death in cells with a high free cholesterol content.

In another embodiment of the above method, the determination of whether the cell is living or non-living comprises contacting the mixture of step (b) with an indicator that specifically binds either living or non-living cells, but not both. In another embodiment, the indicator is a colorometric dye. In another embodiment, the colorometric dye is Trypan Blue, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, nitro blue tetrazolium chloride, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide,

tetrazolium blue chloride, 4-iodonitrotetrazolium violet chloride, or 4-nitrotetrazolium violet chloride.

In another embodiment, the indicator is a fluorescent dye. In another embodiment, the flourescent dye is propidium iodide, YO-PRO-1, SYTO 13, SYTO 16, Hoechst 33342, ethidium bromide, 7-aminoactinomycin D, LDS 751, acridine orange, DAPI, sulforhodamine, ethidium homodimer-2, ethidium monoazide, YOYO-1 SYBR Green I, a SYTOX dye, a cyanine dimer dye or a monomer dye, or any other fluorescent nucleic stain.

In another embodiment, the determination of whether the cell is living or non-living is via an assay. In another embodiment, the assay is a radioactive assay. In another embodiment, the radioactive assay detects ^{51}Cr or ^{3}H-adenine released from cells indicating cell death.

In another embodiment, the radioactive assay detects a radioactive compound preloaded into and retained by a healthy cell.

In another embodiment, the assay is an enzymatic assay. In another embodiment, the enzymatic assay detects the release of an intracellular enzyme. In another embodiment, the intracellular enzyme is lactate dehydrogenase.

In another embodiment, the assay is a bioluminescence

assay. In another embodiment, the bioluminescence assay detects cellular ATP content. In another embodiment, the assay employs a luciferase as a detectable signal.

In another embodiment, the assay is a colorometric assay. In another embodiment, the assay detects DNA damage. In another embodiment, the DNA damage is a DNA strand break..

In another embodiment, the assay is TdT-mediated dUTP nick-end labeling (TUNEL) assay. In another embodiment, the assay detects caspase activity in cells.

In another embodiment, the assay detects release of an intracellular enzyme. In another embodiment, the intracellular enzyme is lactate dehydrogenase.

In another embodiment, the assay detects phosphatidylserine on the outer surface of a cell. In another embodiment, the assay employs a reagent that detects annexin binding to a cell.

In another embodiment, the assay is a fluorescent assay. In another embodiment, the assay detects DNA damage. In another embodiment, the DNA damage is a DNA strand break.

In another embodiment, the assay is TdT-mediated dUTP nick-end labeling (TUNEL) assay, Comet assay, or ChromaTide nucleotides assay.

In another embodiment, the assay detects caspase activity in a cell. In another embodiment, the assay detects phosphatidylserine on the outer surface of a cell. In another embodiment, the assay employs a reagent that detects annexin binding to a cell.

In another embodiment, the assay detects mitochondrial dysfunction. In another embodiment, the assay employs JC-1, a MitoTracker dye, rhodamine 123, a carbocyanine dye, a tetramethylrhodamine dye, calcein AM, or nonyl acridine orange.

In another embodiment, the assay employs a free radical probe. In another embodiment, the free radical probe is 2',7'-dichlorodihydrofluorescein diacetate, dihydrorhodamine 123, or dihydroethidium.

In another embodiment, the assay employs an ion indicator. In another embodiment, the ion indicator is SNARF-1 AM or BCECF AM.

In another embodiment, the assay employs an esterase substrate. In another embodiment, the esterase substrate is carboxyfluorescein diacetate or Oregon Green 488 carboxylic acid diacetate.

In another embodiment, the assay measures oxidation or reduction. In another embodiment, the assay employs resazurin, a dihydrorhodamine, a dihydrofluorescein, RedoxSensor Red CC-1, or a tetrazolium salt.

In another embodiment, the assay detects transmembrane potential gradients. In another embodiment, the assay employs a fast-response styryl dye, a slow-response oxonol dye, a carbocyanine dye, or JC-1.

In another embodiment, the assay detects acidic organelles. In another embodiment, the assay employs neutral red or LysoTracker Green DND-26.

In another embodiment, the assay measures europium released by the cell.

In one embodiment, the compound is a peptide, a peptidomimetic, a nucleic acid, an organic molecule, an inorganic chemical, or a lipid-based compound. In another embodiment, the compound is a small molecule having a molecular weight of less than 5,000 Daltons. The compound may be a molecule having a molecular weight of between 50 Daltons and 300 Daltons. The compound may be a molecule having a molecular weight of between 150 Daltons and 1000 Daltons. The compound may be a molecule having a molecular weight of between 750 Daltons and 8000 Daltons. The compound may be a molecule having a molecular weight of between 7500 Daltons and 15000 Daltons.

In one embodiment of the method described hereinabove, inhibition is effected by the compound inhibiting the function of a cellular protein or lipid critical for

intracellular cholesterol transport. The inhibition may be effected by the compound binding to a cellular protein or lipid critical for intracellular cholesterol transport. Inhibition may occur during transcription or translation or as interference with the function of the protein or lipid. In another embodiment, the protein is npc1, npc2, or vacuolar protein sorting 4 protein (VPS4), or any other cellular protein critical for intracellular cholesterol transport. In another embodiment, the lipid is lysophosphatidic acid.

The present invention provides for a pharmaceutical composition comprising: (i) a compound that inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site determined to do so by the method described hereinabove; and (ii) a carrier.

In one embodiment of the composition described hereinabove, the carrier comprises saline, sodium acetate, ammonium acetate, a virus, a liposome, a microencapsule, a polymer encapsulated cell, a retroviral vector, a diluent, or an isotonic, pharmaceutically acceptable buffer solution, or any other pharmaceutically acceptable carrier.

The present invention provides for a method for preventing plaque rupture or superficial erosion in a subject which comprises administering to the subject a therapeutically effective amount of the pharmaceutical

composition described hereinabove so as to prevent plaque rupture or superficial erosion. In one embodiment, the subject is suffering from atherosclerosis. The subject may be suffering from or predisposed to developing atherosclerosis or an atherosclerosis-associated disorder or condition. In another embodiment, the subject may be suffering from diabetes, renal failure, amyloidoses, aging or inflammation. The subject may be an obese subject as defined by the American Medical Association height and weight standards. The subject may be aged. In one embodiment, the subject is a mammal. The subject may be a human, a primate, an equine subject, an opine subject, an avian subject, a bovine subject, a porcine, a canine, a feline or a murine subject. In one embodiment, the plaque rupture or superficial erosion leads to acute thrombosis, vascular occlusion, stroke, tissue infarction, or other acute vascular disease or condition.

In one embodiment, the compound comprises a peptide, a peptidomimetic, a nucleic acid, an organic molecule, an inorganic chemical, or a lipid-based compound linked to a carrier. In another embodiment, the carrier comprises saline, sodium acetate, ammonium acetate, a virus, a liposome, a microencapsule, a polymer encapsulated cell, a retroviral vector, a diluent, or an isotonic, pharmaceutically acceptable buffer solution. In another embodiment, the subject is a mammal. In another embodiment, the mammal is a human.

The present invention provides for a compound previously unknown that inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site in the cell determined to do so by the methods described hereinabove.

The present invention provides for a method for treating a subject suffering from atherosclerosis which comprises administering to the subject the pharmaceutical compositions or the compounds described hereinabove.

The invention provides for a method for preventing or delaying plaque rupture or superficial erosion in a subject which comprises administering to the subject an effective amount of the pharmaceutical compositions or the compounds described hereinabove so as to prolong the life of a macrophage within plaque lesions which exist in the subject, thereby preventing or delaying plaque rupture or superficial erosion in the subject. In one embodiment, the compound is progesterone or an amphipathic amine.

The present invention provides for a method for identifying a compound which inhibits expression of npc1 which comprises: (a) admixing the compound with a cell which expresses npc1; (b) determining the level of expression of npc1; (c) comparing the level of expression in step (b) with the level expressed in the absence of the compound, a lower level of expression in the presence of the compound than in the absence of the

compound indicating that the compound inhibits expression of npc1. Inhibition may occur during transcription or translation or as interference with the function of the protein.

The present invention provides for a method for identifying a compound which inhibits expression of npc2 which comprises: (a) admixing the compound with a cell which expresses npc2; (b) determining the level of expression of npc2; (c) comparing the level of expression in step (b) with the level expressed in the absence of the compound, a lower level of expression in the presence of the compound than in the absence of the compound indicating that the compound inhibits expression of npc2. Inhibition may occur during transcription or translation or as interference with the function of the protein.

The present invention provides for a method for identifying a compound which inhibits expression of vacuolar protein sorting 4 protein (VPS4) which comprises: (a) admixing the compound with a cell which expresses vacuolar protein sorting 4 protein (VPS4); (b) determining the level of expression of vacuolar protein sorting 4 protein (VPS4); (c) comparing the level of expression in step (b) with the level expressed in the absence of the compound, a lower level of expression in the presence of the compound than in the absence of the compound indicating that the compound inhibits expression of vacuolar protein sorting 4 protein (VPS4).

Inhibition may occur during transcription or translation or as interference with the function of the protein.

In further embodiments of the methods described hereinabove, the administration of the pharmaceutical compositions and the compounds described hereinabove is via intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

The sequence of npc1 has been identified and is disclosed in Carstea, E.D., et al., Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis, Science 277:228-231 (1997), the contents of which are hereby incorporated by reference into this application.

The sequence of VPS4 has been identified and is disclosed in Bishop, N. and P. Woodman, ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking, Mol. Biol. Cell 11:227-239 (2000), the contents of which are hereby incorporated by reference into this application.

The sequence of npc2 has not yet been identified. However, Steinberg, S.J., et al., Complementation studies in Niemann-Pick disease type C indicate the existence of a second group, J. Med. Genet. 31:317-320 (1994) and Vanier, M.T., et al., Genetic heterogeneity

in Niemann-Pick C disease: a study using somatic cell hybridization and linkage analysis, Am. J. Hum. Genet. 58:118-125 (1996), the contents of both of which are hereby incorporated by reference into this application, disclose that a genetic mutation indicates that npc2 exists and may be isolated.

We have shown that partial inhibition of the protein npc1 conveys marked resistance to free cholesterol-induced macrophage death in culture and a decrease in atherosclerotic lesional necrosis *in vivo* (mice). Because macrophage death and lesional necrosis has been associated with and likely precipitates atherosclerotic plaque rupture, which is often the cause of atherosclerosis-associated acute thrombosis and thus acute vascular event, pharmacological inhibition of macrophage death and lesional necrosis, perhaps via partial inhibition of npc1, may represent a novel therapy to prevent these acute vascular events.

In addition to plaque rupture, superficial erosion is often the cause of acute thrombosis and thus acute clinical vascular events as described herein.

This invention is useful as a potential therapy for the prevention of acute vascular clinical events, such as myocardial infarction, aneurism, angina, peripheral vascular disease, stroke, acute occlusive thrombosis or other clinical event associated with atherosclerosis, or other peripheral vascular disease.

The methods, compounds, and compositions described herein are useful in both primary and secondary prevention of plaque rupture and superficial erosion, and simultaneous or subsequent acute clinical vascular events. Primary prevention is directed to a subject who has not yet experienced an acute clinical event, but may be susceptible to or predisposed to plaque rupture or superficial erosion. Administration of the compounds or compositions described herein by the described methods would therefore be useful in preventing future acute vascular clinical events by preventing plaque rupture or superficial erosion in these subjects. Secondary prevention is directed to a subject who has experienced an acute vascular clinical event as described herein, and who is therefore presumed to be susceptible to or predisposed to further plaque rupture or superficial erosion and simultaneous or subsequent acute vascular clinical events. Administration of the compounds or compositions described in the present invention by the described methods would similarly be useful in preventing future acute vascular clinical events in these subjects.

The methods, compounds, and compositions described herein are therefore useful in preventing macrophage death, plaque rupture or superficial erosion, acute thrombosis, and other vascular conditions.

It has been shown that free cholesterol loading causes macrophage death. (G.J. Warner, et al.). Kellner-

Weibel, et al. showed that progesterone and an amphipathic amine stop macrophage death in vitro.

One way in which this invention differs from the prior art is that it is the first study using molecular genetics and showing the effect on lesional necrosis in vivo. This invention therefore provides an advantage over the prior art in that it provides evidence that lesional necrosis can be decreased in vivo.

Methods of preparing various pharmaceutical compositions with a certain amount of active ingredient are known, or will be apparent in light of this disclosure, to those skilled in this art. For examples of methods of preparing pharmaceutical compositions, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 18th Edition (1990).

U.S. Patent No. 6,034,102, the contents of which are hereby incorporated by reference into this application, provides additional information relating to methods for treating atherosclerosis and preparing and administering pharmaceutical compositions and derivatives thereof.

U.S. Patent Nos. 6,043,260 and 6,051,597, the contents of which are hereby incorporated by reference into this application, provide additional information relating to preparing and administering pharmaceutical compositions in the treatment of diseases or conditions.

As used herein, the terms "treating", "treatment", "treat" include curative, preventative (e.g. prophylactic) and palliative treatment.

As used herein, preventing or delaying a plaque rupture or superficial erosion, or simultaneous or subsequent vascular event includes ameliorating, suppressing, halting, slowing the progression of, or controlling the plaque rupture or superficial erosion, or simultaneous or subsequent vascular event.

As used herein, the term "composition", as in pharmaceutical composition, is intended to encompass a product comprising the active ingredient(s) and the inert ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly from combination, complexation, or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients.

As used herein, "effective amount" refers to an amount which is capable of treating or preventing a plaque rupture or superficial erosion or treating or preventing or delaying the onset of a disease or disorder or other clinical event described herein, or preventing or delaying the onset of macrophage death. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated.

Exact dosage and dosing schedules for the administration of the compounds and compositions described hereinabove can be determined by a skilled physician.

As used herein, "pharmaceutically acceptable carrier" means that the carrier is compatible with the other ingredients of the formulation and is not deleterious to the recipient thereof, and encompasses any of the standard pharmaceutically accepted carriers.

Other features and advantages of this invention will be apparent from the specification and claims which describe the invention.

The present invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Example 1: Heterozygous Deficiency of the Npc1 Protein is Associated with a Marked Resistance to Free Cholesterol-Induced Macrophage Death in Culture and to a Selective Decrease inatherosclerotic Lesional Necrosis *In Vivo*

Abstract

Necrotic areas of advanced atheromata are thought to play an important role in the acute clinical events associated with atherosclerotic vascular disease. Previous studies have suggested that macrophage death, perhaps caused by excess cellular FC, may contribute to the formation of these necrotic areas. Herein we explore FC-mediated macrophage death in cell culture and necrotic area formation *in vivo* using the Niemann-Pick C (NPC) mouse model. In this model, a mutation in the npc1 protein results in defective free cholesterol (FC) transport from lysosomes to peripheral membranes. We predicted that this defect, by sequestering FC away from critical membrane proteins, might protect cells from the toxicity of excess cellular FC. In the first set of experiments, wild-type and heterozygous NPC (NPC1) peritoneal macrophages were loaded with FC by incubation for 24 h with acetyl-LDL plus an acyl-CoA:cholesterol acyltransferase inhibitor. The percentage of dead cells under these conditions was 29.7 ± 4.1% for wild-type macrophages but only 8.3 ± 1.0% of NPC1 macrophages.

NPC1 macrophages were equally susceptible to other death inducers, such as serum withdrawal. Next, we examined advanced atherosclerotic lesions in apolipoprotein E knockout (E0) mice, a model of atherosclerosis, in the absence or presence of the NPC1 mutation. In the lesions of E0 mice, there were many areas that were acellular, rich in FC but not cholesteryl esters, and, most importantly, contained macrophage proteins (*i.e.*, "debris"). Remarkably, these necrotic areas were decreased by ~50% ($p = 0.00001$) in NPC1/E0 lesions whereas total atherosclerotic lesion area was decreased by only ~20% in NPC1/E0 mice ($p = 0.05$). In summary, we have shown that a partial deficiency of the npc1 protein leads to a marked resistance to FC-mediated macrophage death in culture and to a selective decrease in necrotic areas in advanced atherosclerotic lesions *in vivo*.

Introduction

A prominent feature of advanced atherosclerotic lesions is the presence of necrotic areas, which are sites inside the thickened intima consisting of cellular debris and extracellular lipid (1,2). The importance of these necrotic areas lies in the fact that they are often found in areas of plaque rupture, which is the most common precipitating cause of atherosclerosis-associated acute thrombosis, vascular occlusion, and tissue infarction (3). Given the potential clinical implications of lesion necrosis, surprisingly little is known about the mechanisms of necrotic area development.

While there is evidence that some of the lipid in these areas is derived directly from extracellular, plasma-derived lipoproteins, cell death with subsequent release of intracellular lipids and other potentially harmful molecules is likely to be a central event (2,4,5). In this regard, recent data with antibodies against cell-type-specific intracellular proteins support the idea that the cholesterol-loaded macrophage, a major cellular constituent of atherosclerotic lesions, is the main cell type that dies in the vicinity of necrotic areas (4,6). The mechanistic link between macrophage death and unstable plaques may be related to plaque-destabilizing enzymes and pro-coagulant/thrombogenic molecules released by these dying cells (7).

The causes of macrophage death in advanced atherosclerosis are not known. Several factors or conditions, such as oxidized lipids, growth factor deprivation, and inflammatory cytokines, have been proposed but not rigorously tested *in vivo* (1,8). Another cytotoxic condition that deserves attention is excess cellular free cholesterol (FC) (9). FC accumulation in lesional foam cells has been well-documented (10, 11, 12, 13), and studies with cultured macrophages have shown that excess cellular FC is a potent inducer of cell death (14, 15). The mechanism of cytotoxicity probably involves integral membrane protein dysfunction resulting from a high cholesterol:phospholipid ratio in the membranes surrounding these molecules (9, 16, 17). This idea was

recently supported by the data of Kellner-Weibel *et al.* (18), who showed that FC-induced cytotoxicity was inhibited by amphipathic amines, which block the transport of lipoprotein-derived FC from lysosomes to peripheral membranes, particularly the plasma membrane. Interestingly, Papahadjopoulos (16) demonstrated twenty-five years ago that FC-mediated inhibition of the plasma membrane proteins Na^+-K^+-ATPase and adenylate cyclase leads to cellular death, and he proposed that these events may play an important role in the development of necrosis in advanced atheromata.

Although cell cultures studies have suggested potentially important ideas related to inducers and mechanisms of macrophage death, little is known about the factors that influence the development of lesional necrosis *in vivo*. We reasoned that an *in-vivo* model might already exist to begin to explore some of these ideas. This model, the Niemann-Pick C (NPC) mouse, like humans with NPC disease, has a mutation in a protein called npc1 that results in a block of FC transport from lysosomes to peripheral cellular sites (19, 20, 21, 22). According to the ideas described above, this specific molecular genetic defect might be expected to protect cells from FC-mediated cytotoxicity. In this report, we show that cultured macrophages derived from NPC mice are, indeed, markedly resistant FC-mediated death. Most remarkably, the atherosclerotic lesions of NPC mice on the apolipoprotein E-knockout (E0) atherosclerotic background have a substantial reduction in necrotic area

despite only a minimal decrease in total lesion area. While future *in-vivo* studies will be required to mechanistically link the cultured macrophage data with the lesional data, these findings show that a specific gene/protein alteration is associated with a selective reduction in atherosclerotic lesional necrosis.

Materials & Methods

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher Scientific Co. (Springfield, NJ). Tissue culture media and other tissue culture reagents were obtained from GIBCO BRL. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT) and was heat-inactivated for 1 h at 65°C (HI-FBS). Compound 58035 (3-[decyldimethylsilyl]-*N*-[2-(4-mrthylphenyl)-1-phenylethyl]propanamide (23), an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT), was generously provided by Dr. John Heider of Sandoz, Inc. (East Hanover, NJ); a 10 mg/ml stock solution was prepared in dimethyl sulfoxide, and the final dimethyl sulfoxide concentration in both treated and control cells was 0.05%. All other chemicals and reagents were from Sigma, and all organic solvents were from Fisher Scientific Co.

Mice—Balb/C mice heterozygous for the NPC mutation (NPC1) were obtained from Dr. Peter Pentchev (National Institutes of Health). These mice were backcrossed into the C57BL/6 background for four generations and then

bred into the E0/C57BL/6 background for an additional generation. Matings of NPC1/E0 x NPC1/E0 were used to generate the E0 and NPC1/E0 mice used in this study. After weaning, the mice were placed on a high-cholesterol diet and sacrificed at 25 weeks of age for atherosclerotic lesion studies (below).

Harvesting and Culturing Mouse Peritoneal Macrophages—Mouse peritoneal macrophages were harvested from the peritoneum of mice 3 days after the intraperitoneal injection of 40 μg of concanavalin A in 0.5 ml of PBS (24). The cells were plated in 22-mm dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS, 20% (v/v) L-cell conditioned medium (LCM), penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (292 μg/ml) and then incubated at 37°C in an atmosphere containing 5% CO_2. When the cells were 70-80% confluent, they were used for the studies described below.

FC-Loading and Cell Death Assay—Monolayers of peritoneal macrophages were washed three times with warm PBS and incubated for the indicated times in 0.5 ml of DMEM/0.2% BSA (w/v) alone or containing 10 μg acetyl-LDL/ml plus 10 μg of compound 58035/ml as previously described (25). At the end of the incubation period, the cells were assayed for cell death by permeability to the fluorescent dye propidium iodide (26). After staining with propidium iodide, the cells were viewed by fluorescence microscopy, and 15 fields of cells for each conditions (~2000 cells) were counted to determine the percentage of propidium iodide-positive cells.

Plasma Lipid and Lipoprotein Assays

Preparation and Staining of Histological Sections

Hearts from E0 and NPC1/E0 mice (above) were perfused, embedded in optimum-cutting-temperature (OCT) compound (Sakura Finetek, Torrance, CA), snap-frozen in ethanol-dry-ice bath and stored at -70°C. Multiple 8-μm-thick sections of murine, rabbit, and human aorta were cut on a cryostat, placed on poly-L-lysine-coated glass slides, and fixed in 10% buffered formalin for 5 min at room temperature. The sections were air-dried for 10-15 min, washed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 20 min, and rinsed in PBS for 5 min in PBS at room temperature. The sections were then preincubated with 2% normal serum in PBS for 1 h at room temperature. Next, the sections were incubated with 2% donkey serum. After the sections were washed in PBS for 5 min, the bound primary antibody was visualized using biotinylated secondary antibody followed by streptavidin peroxidase (Vectastain Elite ABC-peroxidase kit; Vector Laboratories Inc., Burlingame, CA) and 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin, rinsed, mounted in permount, and viewed with an Olympus IX 70 inverted microscope using a 20X objective.

Quantification of Total Atherosclerotic Lesion Area and of Necrotic Area

Statistics--Results are given as means ± S.E.M. For comparisons between a single experimental group and a control, the unpaired, two-tailed t-test was used.

Results

Macrophages from Heterozygous NPC Mice are Resistant to FC-Mediated Cell Death—

To test the idea that a molecular genetic alteration in peripheral cholesterol transport would protect macrophages from FC-mediated cytotoxicity, peritoneal macrophages from wild-type and Niemann-Pick C (NPC) mice (21,22) were loaded with FC for 12 h by incubation with acetylated LDL plus an inhibitor of cholesterol esterification (15). After 12 h of loading, the wild-type macrophages became rounded and started to detach (leading to 30% loss of attached cellular protein), whereas both the NPC1 and NPC0 macrophages were well-spread and remained attached to the plate (no loss of attached cellular protein) (data not displayed). We next compared wild-type and NPC1 macrophages using a more prolonged (24-h) FC-loading protocol. In addition, we employed a more quantitative measurement of cytotoxicity, namely, permeability to the fluorescent compound propidium iodide (PI) (26). Whereas a substantial percentage of the wild-type Mfs stained with PI as expected, the NPC1 macrophages remained mostly PI-impermeable (Fig. 1A). Thus, even a partial defect in FC transport markedly protects macrophages from the toxic effects of prolonged FC loading.

To determine if NPC1 macrophages were protected from other inducers of death, we compared these cells with wild-type macrophages for their susceptibility to

oxidized LDL-induced and serum withdrawal-induced death.

Characterization of Areas of Cell Death in the Atherosclerotic Lesions of E0 Mice—Mice engineered to lack apolipoprotein E (E0 mice) develop extensive atherosclerotic lesions with areas of necrosis (27,28). In preparation for experiments designed to look at the influence of the NPC mutation on lesional cell death (below), we characterized in detail the necrotic-appearing areas in advanced lesions of E0 mice. As shown in Fig. 2A, raised lesions from the proximal aorta of 25-week-old E0 mice contained acellular areas situated beneath a layer of endothelial and intimal cells (the *arrow* in Fig. 2 depicts one of these areas). Using a stain for collagen, we focused on acellular areas that were not simply dense fibrous scars (not shown). Next, because extracellular FC accumulation is a property of necrotic areas of advanced atherosclerotic lesions (1, 2, 29), we stained the lesions with filipin, a fluorescent dye that binds FC (29). As demonstrated in Fig. 2B, most of the acellular areas, as well many of the cellular areas of the intima, bound filipin, whereas the outer layer of the lesion bound no filipin. Importantly, the acellular areas of E0 lesions stained only weakly with the neutral lipid dye Oil Red O compared with the cholesteryl ester-rich foam cell areas (see below), indicating that the acellular areas were richer in FC than cholesteryl esters. To determine if these acellular areas might represent sites of

macrophage death, we looked for the presence macrophage proteins (*i.e.*, debris). Fig. 2C shows the result obtained when the section was immunostained for the macrophage type A scavenger receptor; Fig. 2D is the control using a nonimmune primary antibody. Remarkably, many of the acellular regions stain for this macrophage protein. Similar results were obtained using an antibody directed against the macrophage protein Mac-3 (not shown). Thus, we have demonstrated that the advanced lesions of E0 mice contain acellular areas that stain for both FC and macrophage proteins, suggesting that these areas are, indeed, necrotic areas containing the debris of macrophages.

Analysis of the Lesions of NPC1/E0 Mice—E0 mice (in the C57BL/6 background) and NPC1 mice backcrossed for five generations to the E0/C57BL/6 background (NPC1/E0 mice) were fed a high-cholesterol ("Western") diet for 25 weeks. Both groups of mice appeared normal, and their weights at the end of the 25-week period were not statistically different (not shown). The plasma cholesterol and phospholipid values were also not statistically different between the two groups (Fig. 3A). Similarly, the gel-filtration profiles of the plasma lipoproteins were very similar (Fig. 3B); neither the increase in the VLDL peak nor the decrease in the LDL peak in the NPC1/E0 plasma shown in this figure was reproducible in repeated experiments.

As shown in Fig. 4A, total lesion area in the proximal

aorta of NPC1/E0 mice (n = 9) was decreased ~20% compared with E0 mice (n = 26; p = 0.05). Despite this modest affect on lesion size, the necrotic areas of the NPC1/E0 lesions, as defined using some of the criteria described above, was decreased by ~50% (Fig. 4B; p = 0.00001). Therefore, even when the data were expressed as percent necrotic area, there was a substantial, highly statistically significant difference between the two groups of mice (Fig. 4C). A separate analysis of male and female mice showed the decrease in necrotic area in NPC1 mice was not gender-specific (data not shown); in fact, NPC1/E0 male mice (n = 6) had a 45% decrease in necrotic area compared with male E0 mice (n = 12; p = 0.0002) despite no statistically significant difference in total lesion size.

An example of a histological section from each type of mouse is shown in Fig. 5. Panel A shows extensive acellular areas in the lesion of an E0 mouse, and panel A' demonstrates that these acellular areas stained more weakly for Oil Red O than the foam cell areas, as described above. In striking contrast, the lesion of the NPC1/E0 mice shown in panel B is more cellular and, as expected for a foam cell-rich lesion, is more Oil Red O-positive (panel B'). Thus, the proximal aortic lesions of 25-week-old NPC1/E0 mice have less extensive necrotic area development than those of E0 mice.

Discussion

The data in this report reveal two important properties related to the NPC mutation. First, macrophages from mice with only a heterozygous defect in the *npc* protein show a marked resistance to death resulting from FC loading. These macrophages are not resistant to other inducers of cell death, and so we presume the protection is related specifically to FC-mediated toxicity. Investigators have postulated that excess FC kills cells via inhibition of certain critical plasma membrane enzymes by a high FC:phospholipid ratio in the vicinity of these molecules (9, 16, 17). At a normal FC:phospholipid ratio, membranes contain areas of phospholipid "packing defects" that provide "space" for integral membrane proteins to undergo conformational changes. In the presence of excess FC, however, these packing defects diminish, which restricts the conformational freedom of membrane proteins and inhibits their ability to function properly (17). Decreases in function of critical membrane proteins would then lead to cell sickness and death. FC loading may also lead to mitochondrial dysfunction, another trigger in cell death (30), perhaps by saturation of mitochondrial membranes with excess FC. Recall that FC is known to be trafficked to the mitochondria in several cell types, including macrophages (31, 32). Kellner-Weibel *et al.* (18) showed that amphipathic amines, which partially block FC transport out of lysosomes, prevent FC-mediated toxicity in macrophages, which is consistent with the

concept that FC transport to the plasma membrane and possibly mitochondria is essential for the death response. The data in this report demonstrate this point without the use of these drugs, which may have other effects on cells, and show that the protection is still marked with only a partial defect in FC transport (33). Thus, in heterozygous NPC macrophages, a critical FC:phospholipid ratio threshold may never be reached under the conditions of our experiments.

The second major finding in this report is the effect of the NPC mutation on the characteristics of atherosclerotic lesions of E0 mice. Under conditions of similar plasma cholesterol levels and lipoprotein profiles, lesion size *per se* was only minimally affected, but the area of regions with the characteristics of necrosis was substantially decreased in the lesions of NPC1/E0 mice. As mentioned in the Introduction, there is evidence that macrophage death contributes to the development of lesional necrotic areas, which have been called "a graveyard of dead macrophages" (34). For example, necrotic areas of human lesions have been shown to contain macrophage proteins (4, 6), consistent with our data with E0 mice presented here (Fig. 2), and dying macrophages are often found in the vicinity of necrotic areas of lesions (1, 2, 7). This information, together with the increase in cellularity of the E0/NPC1 lesions (Fig. 5), suggests that at least one reason for the decrease in necrotic area in these lesions is a decrease in macrophage death.

If so, it is tempting to speculate that the decrease in lesional macrophage death is related to the marked resistance of NPC1 macrophages to FC-induced death (Fig. 1). Indeed, macrophages in advanced lesions have been shown to accumulate large amounts of FC (10, 11, 12, 13) which is consistent with our filipin-staining data in Fig. 2, and so FC-induced cytotoxicity is a plausible mechanism for lesional macrophage death (16). The further testing of this hypothesis will be a focus of our future work in this area.

An issue not addressed in this study is whether FC-mediated death is due to apoptosis or necrosis (35). While the distinction between these two modes of death may not always be clear (36, 37), Rothblat and colleagues (18) have presented preliminary evidence that FC loading of macrophages results in the appearance of some apoptotic features in the cells. Using a variety of assays, we have recently shown that FC loading leads to an early apoptotic response followed by later necrotic changes (Yao *et al.*, manuscript in preparation). Because necrotic as well as apoptotic features are decreased in FC-loaded macrophages from NPC1 mice (data not shown), we conclude that normal peripheral FC transport is required for both forms of death. Of note, macrophage death in atherosclerotic lesions shows features of both apoptosis and necrosis (1).

While more studies are needed to mechanistically link

the cell-culture and *in-vivo* data reported herein, the significance of our findings lies in the potential clinical importance of the lesional necrosis in atherosclerotic vascular disease. Atherosclerotic lesions rich in necrotic areas are often referred to as "culprit" lesions because they are susceptible to plaque rupture, which precipitates acute thrombosis and thus acute ischemic events (3). In this regard, it is likely that dying cells release or expose plaque-destabilizing enzymes (*e.g.*, lysosomal proteases and matrix metalloproteinases) and pro-coagulant/thrombogenic molecules (*e.g.*, tissue factor and phosphatidylserine) and thus contribute to plaque rupture and acute thrombosis (7). Indeed, Bauriedel *et al.* (5) have reported that atherectomy specimens from patients with unstable angina have approximately twice the number of dead intimal cells compared with specimens from patients with stable angina. Having revealed in this report a specific gene/protein alteration that results in a selective decrease in necrotic area formation, we hope to gain further insight into this critical lesional event. On a specific note, the findings reported herein raise the interesting issue as to whether humans heterozygous for the NPC mutation have a lower incidence of acute ischemic events compared to individuals without this mutation.

Example 2

By way of brief introduction, there is good evidence that acute clinical vascular events result from plaque rupture and that macrophage death in lesions contributes to plaque rupture. One of the causes of lesional macrophage death is likely to be excess cholesterol accumulation in the cell. We have shown that in order for excess cholesterol to kill macrophages, it must be transported from lysosomes to the plasma membrane and other peripheral sites. Pharmacologic or genetic blockage of this transport process has been shown to protect macrophages in culture from cholesterol-induced death, and we have evidence that this transport blockage also prevents macrophage death in lesions *in vivo*.

In essence, therefore, the invention provides a method for treating a subject suffering from advanced atherosclerosis, both before the occurrence of acute clinical events (*i.e.*, primary prevention) and after such events (*i.e.*, secondary prevention). This would comprise administering an inhibitor of intracellular cholesterol transport to the subject to prevent lesional macrophage death. Several known compounds, such as progesterone and various amphipathic amines, are already known to do this in cultured macrophages *in vitro*. In addition, the invention would provide a method for screening new inhibitors of intracellular cholesterol

transport. This method would comprise the following high-throughput screening assay: (a) adding a library of compounds or derivatives of those compounds to monolayers of cultured macrophages in multi-well dishes; (b) adding one of several available toxins, such as amphotericin B, that kills cells only if the content of cholesterol in the plasma membrane is above a certain level; (c) staining dead cells with one of several available colorometric (*e.g.*, Trypan Blue) or fluorescent (e.g., propidium iodide) dyes that do not stain live cells; and (d) colorometric or fluorescent identification of non-staining cells (*i.e.*, those cells that survived because they have been exposed to an inhibitor of cholesterol transport to the plasma membrane). The cellular target of such inhibitors might be the protein npc1 or other protein or lipid targets in the cell that may be critical for transport of cholesterol to the plasma membrane.

It should be noted that this invention is not limited to the particular embodiments described herein, but that various changes and modifications may be made without departing from the spirit and scope of this novel concept as defined by the claims which follow.

References

1. Mitchinson, M.J., S.J. Hardwick, and M.R. Bennett. 1996. Cell death in atherosclerotic plaques. *Curr. Opin. Lipidol.* 7:324-329.

2. Guyton, J.R. and K.F. Klemp. 1996. Development of the lipid-rich core in human atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 16:4-11.

3. Fuster, V.,L. Badimon, J.J. Badimon, and J.H. Chesebro. 1992. The pathogenesis of coronary artery disease and the acute coronary syndromes. *N. Eng. J. Med.* 326:242-250.

4. Ball, R.Y., E.C. Stowers, J.H. Burton, N.R. Cary, J.N. Skepper, and M.J. Mitchinson. 1995. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis* 114:45-54.

5. Bauriedel, G.,I. Schmucking, R. Hutter, and T. Schmidt. 1999. Increased apoptotic and necrotic cell death in human coronary plaques with unstable versus stable angina. *Circulation* 96:I-190(Abstr.)

6. Berberian, P.A., W. Myers, M. Tytell, V. Challa, and M.G. Bond. 1990. Immunohistochemical localization of heat shock protein-70 in normal-appearing and atherosclerotic specimens of human arteries. *Am. J. Pathol.* 136:71-80.

7. Libby, P. and S.K. Clinton. 1993. The role of macrophages in atherogenesis. *Curr. Opin. Lipidol.* 4:355-363.

8. Lopes-Virella, M.F. and G. Virella. 1992. Immune mechanisms of atherosclerosis in diabetes mellitus. *Diabetes* 41:Suppl 2:86-91.

9. Tabas, I. 1997. Free cholesterol-induced cytotoxicity. A possible contributing factor to macrophage foam cell necrosis in advanced atherosclerotic lesions. *Trends Cardiovasc. Med.* 7:256-263.

10. Lundberg, B. 1985. Chemical composition and physical state of lipid deposits in atherosclerosis. *Atherosclerosis* 56:93-110.

11. Small, D.M., M.G. Bond, D. Waugh, M. Prack, and J.K. Sawyer. 1984. Physicochemical and histological changes in the arterial wall of nonhuman primates during progression and regression of atherosclerosis. *J. Clin. Invest.* 73:1590-1605.

12. Rapp, J.H., W.E. Connor, D.S. Lin, T. Inahara, and J.M. Porter. 1983. Lipids of human atherosclerotic plaques and xanthomas: clues to the mechanism of plaque progression. *J. Lipid Res.* 24:1329-1335.

13. Shio, H., N.J. Haley, and S. Fowler. 1979.

Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. III. Intracellular localization of cholesterol and cholesteryl ester. *Lab. Invest.* 41:160-167.

14. Warner, G.J., G. Stoudt, M. Bamberger, W.J. Johnson, and G.H. Rothblat. 1995. Cell toxicity induced by inhibition of acyl coenzyme A: cholesterol acyltransferase and accumulation of unesterified cholesterol. *J. Biol. Chem.* 270:5772-5778.

15. Tabas, I., S. Marathe, G.A. Keesler, N. Beatini, and Y. Shiratori. 1996. Evidence that the initial up-regulation of phosphatidylcholine biosynthesis in free cholesterol-loaded macrophages is an adaptive response that prevents cholesterol-induced cellular necrosis. Proposed role of an eventual failure of this response in foam cell necrosis in advanced atherosclerosis. *J. Biol. chem.* 271:22773-22781.

16. Papahadjopoulos, D. 1974. Cholesterol and cell membrane function: a hypothesis concerning the etiology of atherosclerosis. *J. Theor. Biol.* 43:329-337.

17. Yeagle, P.L. 1991. Modulation of membrane function by cholesterol. *Biochimie* 73:1303-1310.

18. Kellner-Weibel, G., W.G. Jerome, D.M. Small, G.J. Warner, J.K. Stoltenborg, M.A. Kearney, M.H. Corjay, M.C. Phillips, and G.H. Rothblat. 1998. Effects of

intracellular free cholesterol accumulation on macrophage viability: a model for foam cell death. *Arterioscler. Thromb. Vasc. Biol.* 18:423-431.

19. Pentchev, P.G., A.D. Boothe, H.S. Kruth, H. Weintroub, J. Stivers, and R.O. Brady. 1984. A genetic storage disorder in BALB/C mice with a metabolic block in esterification of exogenous cholesterol. *J. Biol. Chem.* 259:5784-5791.

20. Loftus, S.K., J.A. Morris, E.D. Carstea, J.Z. Gu, C. Cummings, A. Brown, J. Ellison, K. Ohno, M.A. Rosenfeld, D.A. Tagle, P.G. Pentchev, and W.J. Pavan. 1997. Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science* 277:232-235.

21. Liscum, L. and J.J. Klansek. 1998. Niemann-Pick disease type C. *Curr. Opin. Lipidol.* 9:131-135.

22. Pentchev, P.G., E.J. Blanchette-Mackie, and L. Liscum. 1997. Biological implications of the Niemann-Pick C mutation. *Sub-Cell. Biochem.* 28:437-451.

23. Ross, A.C., K.J. Go, J.G. Heider, and G.H. Rothblat. 1984. Selective inhibition of acyl coenzyme A:cholesterol acyltransferase by compound 58-035. *J. Biol. Chem.* 259:815-819.

24. Tang, W.,A. Walsh, and I. Tabas. 1999. Macrophage-targeted CTP: phosphocholine

cytidylyltransferase (1-314) transgenic mice. *Biochim. Biophys. Acta* 1437:301-316.

25. Shiratori, Y., A.K. Okwu, and I. Tabas. 1994. Free cholesterol loading of macrophages stimulates phosphatidylcholine biosynthesis and up-regulation of CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* 269:11337-11348.

26. Ross, D.D., C.C. Joneckis, J.V. Ordonez, A.M. Sisk, R.K. Wu, A.W. Hamburger, Nora RE, and R.E. Nora. 1989. Estimation of cell survival by flow cytometric quantification of fluorescein diacetate/propidium iodide viable cell number. *Cancer Res.* 49:3776-3782.

27. Nakashima, Y., A.S. Plump, E.W. Raines, J.L. Breslow, and R. Ross. 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* 14:133-140.

28. Reddick, R.L., S.H. Zhang, and N. Maeda. 1994. Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. *Arterioscler. Thromb.* 14:141-147.

29. Kruth, H.S. 1985. Subendothelial accumulation of unesterified cholesterol. An early event in atherosclerotic lesion development. *Atherosclerosis* 57:337-341.

30. Green, D.R. 1998. Apoptotic pathways. *Cell* 94:695-698.

31. Bjorkhem, I., O. Andersson, U. Diczfalusy, B. Sevastik, R.J. Xiu, C. Duan, and E. Lund. 1994. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc Natl Acad Sci USA* 91:8592-8596.

32. Liscum, L. and N.K. Dahl. 1992. Intracellular cholesterol transport. *J. Lipid Res.* 33:1239-1254.

33. Kruth, H.S., M.E. Comly, J.D. Butler, M.T. Vanier, J.K. Fink, D.A. Wenger, S. Patel, and P.G. Pentchev. 1986. Type C Niemann-Pick disease. Abnormal metabolism of low density lipoprotein in homozygous and heterozygous fibroblasts. *J. Biol. Chem.* 261:16769-16774.

34. Schaefer, H.E. 1981. The role of macrophages in atherosclerosis. F. Schmalze, D. Huhn, and H.E. Schaefer, editors. Disorders of the Monocyte-Macrophage System. 137-142.

35. Arends, M.J. and A.H. Wyllie. 1991. Apoptosis: mechanisms and roles in pathology. *Int. Rev. Exper. Pathol.* 32:223-254.

36. Majno, G. and I. Joris. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am.*

J. Pathol. 146:3-15.

37. Zhang, C.,Y. Xu, J. Gu, and S.F. Schlossman. 1998. A cell surface receptor defined by a mAb mediates a unique type of cell death similar to oncosis. *Proc. Natl. Acad. Sci. USA* 95:6290-6295.

What is claimed is:

1. A method of determining whether a compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell which comprises:
   (a) admixing the compound with a cell;
   (b) contacting the mixture in (a) with a toxin that causes cell death only if excess cholesterol is present at the peripheral site;
   (c) determining whether the cell either is living or non-living, wherein a living cell indicates that the compound inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site within the cell.

2. The method of claim 1 wherein the intracellular cholesterol storage site is a lysosome, a recycling endosome, a sorting endosome, or a late endosome.

3. The method of claim 1, wherein the peripheral site is a plasma membrane of the cell or a mitochondria, an endoplasmic reticulum, a peroxisome, nucleus or a Golgi apparatus in the cell.

4. The method of claim 1, wherein the cell is a

macrophage, an endothelial cell, a smooth muscle cell, a T cell, a dendritic cell, or another arterial-wall cell.

5. The method of claim 1, wherein the toxin in (b) is amphotericin B, filipin, streptolysin O, pneumolysin, perfringolysin O (theta toxin), Vibrio cholerae cytolysin, aerolysin, Listeriolysin O, Vibrio vulnificus haemolysin (VVH), staphylococcal alpha toxin, Aeromonas hydrophilia cytotoxic endotoxin (ACT) or any derivative thereof.

6. The method of claim 1, wherein the determination of whether the cell is living or non-living comprises contacting the mixture of step (b) of claim 1 with an indicator that specifically binds either living or non-living cells, but not both.

7. The method of claim 6, wherein the indicator is a colorometric dye.

8. The method of claim 7, wherein the colorometric dye is Trypan Blue, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, nitro blue tetrazolium chloride, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, tetrazolium blue chloride, 4-iodonitrotetrazolium violet chloride, or 4-nitrotetrazolium violet chloride.

9. The method of claim 6, wherein the indicator is a fluorescent dye.

10. The method of claim 9, wherein the fluorescent dye is propidium iodide, YO-PRO-1, SYTO 13, SYTO 16, Hoechst 33342, ethidium bromide, 7-aminoactinomycin D, LDS 751, acridine orange, DAPI, sulforhodamine, ethidium homodimer-2, ethidium moncazide, YOYO-1 SYBR Green I, a SYTOX dye, a cyanine dimer dye or a monomer dye.

11. The method of claim 1, wherein the determination of whether the cell is living or non-living is via an assay.

12. The method of claim 11, wherein the assay is a radioactive assay.

13. The method of claim 12, wherein the radioactive assay detects ^{51}Cr or ^{3}H-adenine released from cells indicating cell death.

14. The method of claim 12, wherein the radioactive assay detects a radioactive compound preloaded into and retained by a healthy cell.

15. The method of claim 11, wherein the assay is an enzymatic assay.

16. The method of claim 15, wherein the enzymatic

assay detects the release of an intracellular enzyme.

17. The method of claim 16, wherein the intracellular enzyme is lactate dehydrogenase.

18. The method of claim 11, wherein the assay is a bioluminescence assay.

19. The method of claim 18, wherein the bioluminescence assay detects cellular ATP content.

20. The method of claim 18, wherein the assay employs a luciferase as a detectable signal.

21. The method of claim 11, wherein the assay is a colorometric assay.

22. The method of claim 21, wherein the assay detects DNA damage.

23. The method of claim 22, wherein the DNA damage is a DNA strand break.

24. The method of claim 21, wherein the assay is TdT-mediated dUTP nick-end labeling (TUNEL) assay.

25. The method of claim 21, wherein the assay detects caspase activity in cells.

26. The method of claim 21, wherein the assay detects release of an intracellular enzyme.

27. The method of claim 26, wherein the intracellular enzyme is lactate dehydrogenase.

28. The method of claim 21, wherein the assay detects phosphatidylserine on the outer surface of a cell.

29. The method of claim 28, wherein the assay employs a reagent that detects annexin binding to a cell.

30. The method of claim 11, wherein the assay is a fluorescent assay.

31. The method of claim 30, wherein the assay detects DNA damage.

32. The method of claim 31, wherein the DNA damage is a DNA strand break.

33. The method of claim 30, wherein the assay is TdT-mediated dUTP nick-end labeling (TUNEL) assay, Comet assay, or ChromaTide nucleotides assay.

34. The method of claim 30, wherein the assay detects caspase activity in a cell.

35. The method of claim 30, wherein the assay detects phosphatidylserine on the outer surface of a cell.

36. The method of claim 35, wherein the assay employs a reagent that detects annexin binding to a cell.

37. The method of claim 30, wherein the assay detects mitochondrial dysfunction.

38. The method of claim 37, wherein the assay employs JC-1, a MitoTracker dye, rhodamine 123, a carbocyanine dye, a tetramethylrhodamine dye, calcein AM, or nonyl acridine orange.

39. The method of claim 30, wherein the assay employs a free radical probe.

40. The method of claim 39, wherein the free radical probe is 2',7'-dichlorodihydrofluorescein diacetate, dihydrorhodamine 123, or dihydroethidium.

41. The method of claim 30, wherein the assay employs an ion indicator.

42. The method of claim 41, wherein the ion indicator is SNARF-1 AM or BCECF AM.

43. The method of claim 30, wherein the assay employs an esterase substrate.

44. The method of claim 43, wherein the esterase substrate is carboxyfluorescein diacetate or

Oregon Green 488 carboxylic acid diacetate.

45. The method of claim 30, wherein the assay measures oxidation or reduction.

46. The method of claim 45, wherein the assay employs resazurin, a dihydrorhodamine, a dihydrofluorescein, RedoxSensor Red CC-1, or a tetrazolium salt.

47. The method of claim 30, wherein the assay detects transmembrane potential gradients.

48. The method of claim 47, wherein the assay employs a fast-response styryl dye, a slow-response oxonol dye, a carbocyanine dye, or JC-1.

49. The method of claim 30, wherein the assay detects acidic organelles.

50. The method of claim 49, wherein the assay employs neutral red or LysoTracker Green DND-26.

51. The method of claim 30, wherein the assay measures europium released by the cell.

52. The method of claim 1, wherein the compound is a peptide, a peptidomimetic, a nucleic acid, an organic molecule, an inorganic chemical, or a lipid-based compound.

53. The method of claim 1, wherein the compound is a small molecule having a molecular weight of less than 5,000 Daltons.

54. The method of claim 1, wherein inhibition is effected by the compound inhibiting the function of a cellular protein or lipid critical for intracellular cholesterol transport.

55. The method of claim 54, wherein the protein is npc1, npc2, or vacuolar protein sorting 4 protein (VPS4).

56. The method of claim 54, wherein the lipid is lysophosphatidic acid.

57. A pharmaceutical composition comprising:

(i) a compound that inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site determined to do so by the method of claim 1; and

(ii) a carrier.

58. The pharmaceutical composition of claim 57, wherein the carrier comprises saline, sodium acetate, ammonium acetate, a virus, a liposome, a microencapsule, a polymer encapsulated cell, a retroviral vector, a diluent, or an isotonic, pharmaceutically acceptable buffer solution.

59. A method for preventing plaque rupture or superficial erosion in a subject which comprises administering to the subject a therapeutically effective amount of the pharmaceutical composition of claim 57 so as to prevent plaque rupture or superficial erosion.

60. The method of claim 59, wherein the subject is suffering from atherosclerosis.

61. The method of claim 59, wherein the plaque rupture or superficial erosion leads to acute thrombosis, vascular occlusion, stroke, tissue infarction, or other acute vascular disease or condition.

62. The method of claim 1, wherein the compound comprises a peptide, a peptidomimetic, a nucleic acid, an organic molecule, an inorganic chemical, or a lipid-based compound linked to a carrier.

63. The method of claim 57 or 62, wherein the carrier comprises saline, sodium acetate, ammonium acetate, a virus, a liposome, a microencapsule, a polymer encapsulated cell, a retroviral vector, a diluent, or an isotonic, pharmaceutically acceptable buffer solution.

64. The method of claim 59, wherein the subject is a mammal.

65. The method of claim 64, wherein the mammal is a human.

66. A compound previously unknown that inhibits intracellular transport of cholesterol from an intracellular cholesterol storage site to a peripheral site in the cell determined to do so by the method of claim 1.

67. A method for treating a subject suffering from atherosclerosis which comprises administering to the subject the pharmaceutical composition of claim 57 or the compound of claim 62.

68. A method for preventing or delaying plaque rupture or superficial erosion in a subject which comprises administering to the subject an effective amount of the pharmaceutical composition of claim 57 or the compound of claim 62 so as to prolong the life of a macrophage within plaque lesions which exist in the subject, thereby preventing or delaying plaque rupture or superficial erosion in the subject.

69. The method of claim 68, wherein the compound is progesterone or an amphipathic amine.

70. A method for identifying a compound which inhibits expression of npc1 which comprises:

(a) admixing the compound with a cell which

expresses npc1;

(b) determining the level of expression of npc1;

(c) comparing the level of expression in step (b) with the level expressed in the absence of the compound, a lower level of expression in the presence of the compound than in the absence of the compound indicating that the compound inhibits expression of npc1.

71. A method for identifying a compound which inhibits expression of npc2 which comprises:

(a) admixing the compound with a cell which expresses npc2;

(b) determining the level of expression of npc2;

(c) comparing the level of expression in step (b) with the level expressed in the absence of the compound, a lower level of expression in the presence of the compound than in the absence of the compound indicating that the compound inhibits expression of npc2.

72. A method for identifying a compound which inhibits expression of vacuolar protein sorting 4 protein (VPS4) which comprises:

(a) admixing the compound with a cell which expresses vacuolar protein sorting 4

protein (VPS4);

(b) determining the level of expression of vacuolar protein sorting 4 protein (VPS4);

(c) comparing the level of expression in step (b) with the level expressed in the absence of the compound, a lower level of expression in the presence of the compound than in the absence of the compound indicating that the compound inhibits expression of vacuolar protein sorting 4 protein (VPS4).

73. The method of claim 59, 67, or 68, wherein the administration is via intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

FIGURE 1

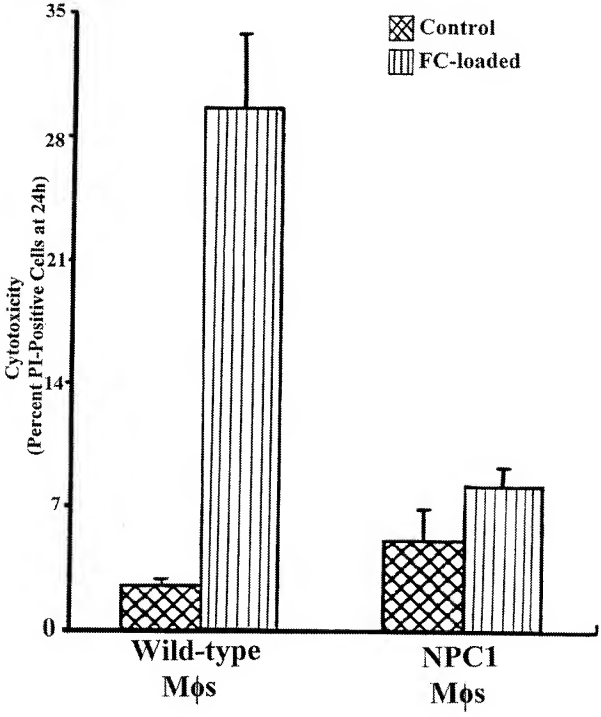
35
28
21
14
7
0
Cytotoxicity
(Percent PI-Positive Cells at 24h)
Control
FC-loaded
Wild-type
Mϕs
NPC1
Mϕs

FIGURE 2A

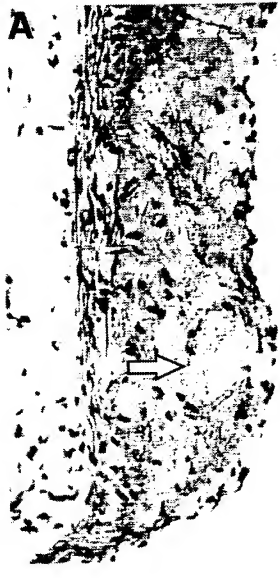

FIGURE 2B

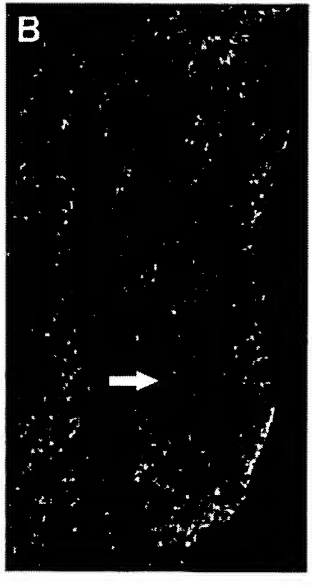

FIGURE 2C

FIGURE 2D

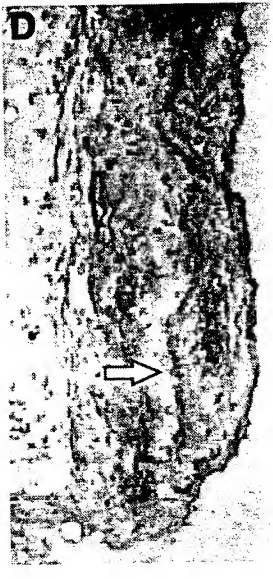

FIGURE 3A

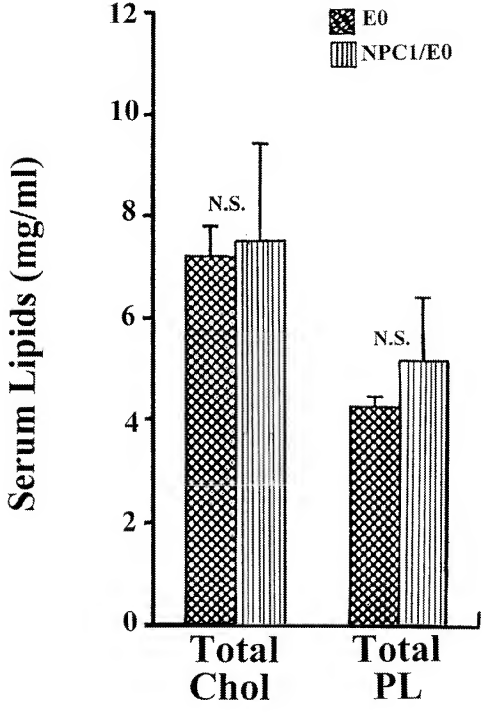
E0
NPC1/E0
Serum Lipids (mg/ml)
12
10
8
6
4
2
0
N.S.
N.S.
Total Chol
Total PL

FIGURE 3B

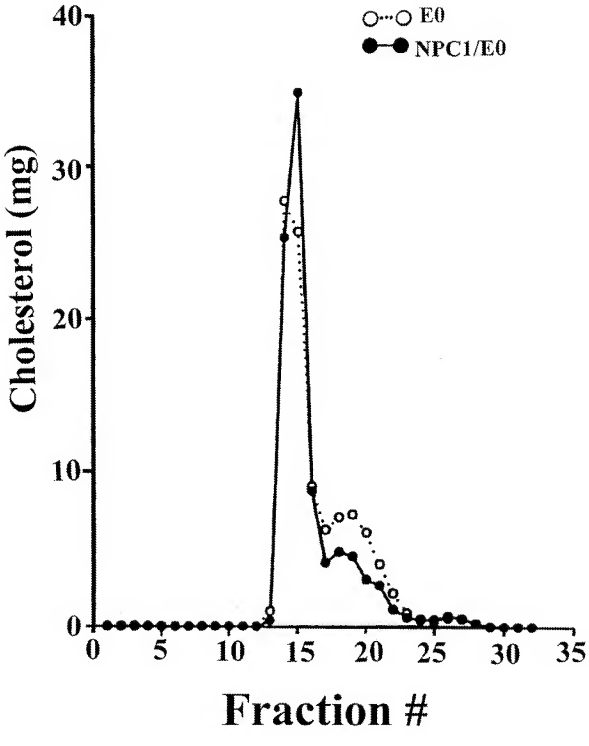
E0
NPC1/E0
Cholesterol (mg)
40
30
20
10
0
0
5
10
15
20
25
30
35
Fraction #

FIGURE 4A

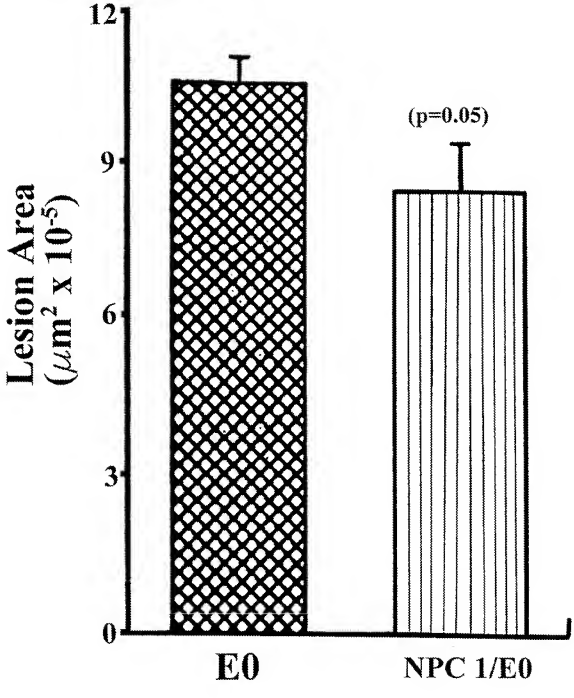

FIGURE 4B

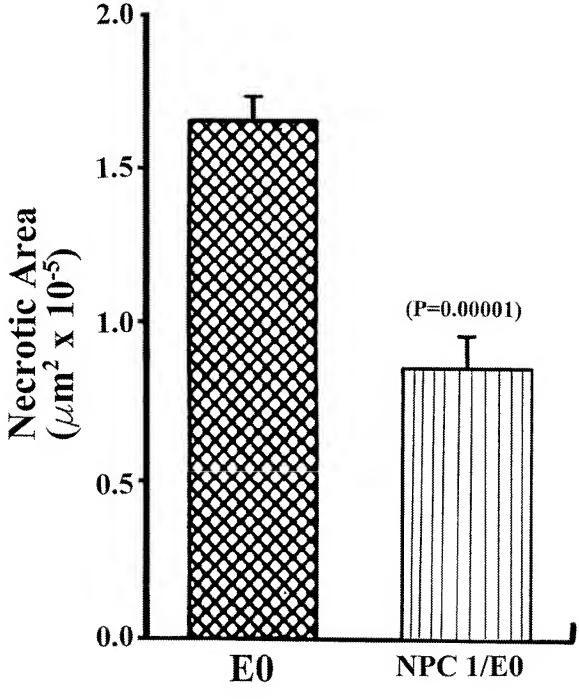

FIGURE 4B

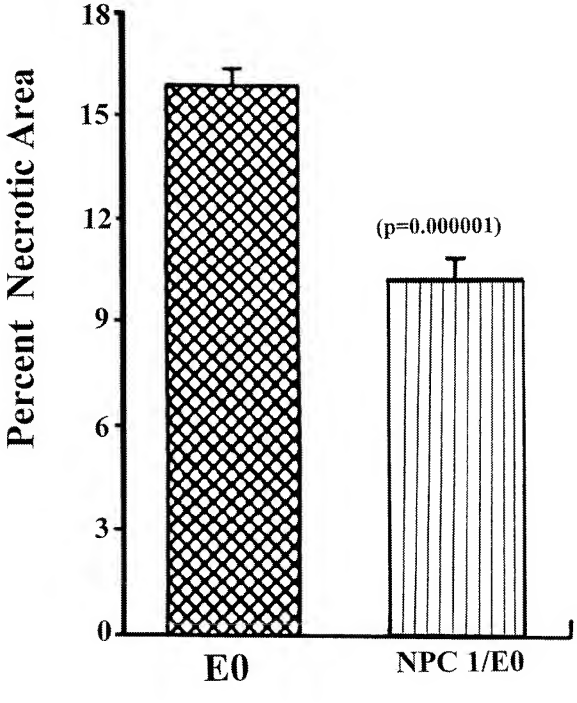

FIGURE 5A

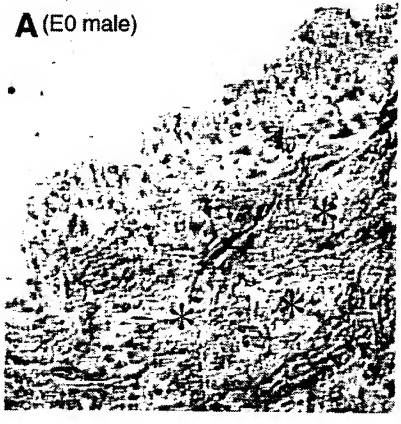

FIGURE 5B

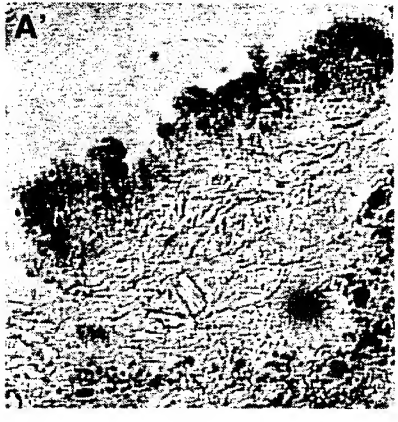

FIGURE 5C

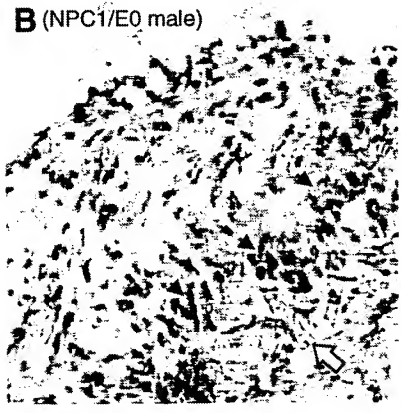

FIGURE 5D